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# Macrophage EP4 Deficiency Increases Apoptosis and Suppresses Early Atherosclerosis

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## SUMMARY

**Prostaglandin (PG) $E_2$, a major product of activated macrophages, has been implicated in atherosclerosis and plaque rupture. The $PGE_2$ receptors, EP2 and EP4, are expressed in atherosclerotic lesions and are known to inhibit apoptosis in cancer cells. To examine the roles of macrophage EP4 and EP2 in apoptosis and early atherosclerosis, fetal liver cell transplantation was used to generate $LDLR^{-/-}$ mice chimeric for $EP2^{-/-}$ or $EP4^{-/-}$ hematopoietic cells. After 8 weeks on a Western diet, $EP4^{-/-} \rightarrow LDLR^{-/-}$ mice, but not $EP2^{-/-} \rightarrow LDLR^{-/-}$ mice, had significantly reduced aortic atherosclerosis with increased apoptotic cells in the lesions. $EP4^{-/-}$ peritoneal macrophages had increased sensitivity to proapoptotic stimuli, including palmitic acid and free cholesterol loading, which was accompanied by suppression of activity of p-Akt, p-Bad, and NF-κB-regulated genes. Thus, EP4 deficiency inhibits the PI3K/Akt and NF-κB pathways compromising macrophage survival and suppressing early atherosclerosis, identifying macrophage EP4-signaling pathways as molecular targets for modulating the development of atherosclerosis.**

## INTRODUCTION

Atherosclerosis, the underlying cause of coronary heart disease and stroke, is a complex degenerative process including an important inflammatory component (Ross, 1999). All of the major cell types involved in atherogenesis produce eicosanoids, and mounting evidence supports critical roles for the prostaglandins and their specific receptors in atherogenesis and plaque stability. Much attention has been given to the roles of prostacyclin ($PGI_2$) and thromboxane A2 ($TXA_2$) and their receptors in atherothrombosis (Grosser et al., 2006). Genetic deletion of the $PGI_2$ receptor accelerates the neointimal proliferation in response to vascular injury (Cheng et al., 2002), whereas deletion of the $TXA_2$ receptor reduces atherosclerosis in apoE-null mice (Kobayashi et al., 2004). Although eicosanoids have been implicated in atherogenesis and plaque stability (Egan et al., 2005; Linton and Fazio, 2008), the roles of $PGE_2$ and its specific prostanoid receptors in atherogenesis have not been examined directly.

Macrophages are the major cell type of early atherosclerotic lesions, and their activation by a variety of stimuli results in abundant COX-mediated production of $PGE_2$. Acting in an autocrine and paracrine manner, $PGE_2$ modulates inflammatory responses via a family of four membrane-spanning G protein-coupled receptors termed EP1, EP2, EP3, and EP4 (Breyer et al., 2001). Human atherosclerotic plaque cells have been reported to express EP4 and EP2 (Gómez-Hernández et al., 2006). Interestingly, platelet EP3 expression has recently been shown to promote thrombosis in response to vascular $PGE_2$ production induced by injury of carotid arteries in apoE-deficient mice (Gross et al., 2007). Deletion of EP2 promotes salt-sensitive hypertension in mice fed a very high sodium diet (Kennedy et al., 1999), whereas EP4 has been shown to be the primary mediator of anti-inflammatory effects of $PGE_2$ and a regulator of chemokine production in vitro (Takayama et al., 2002, 2006). The EP4 receptor has been reported to be responsible for the $PGE_2$-mediated increase in matrix metalloproteinases (MMPs) in macrophages (Pavlovic et al., 2006) that may initiate unstable plaques. Together, these data suggest that macrophage EP2 and EP4 receptor activity may play important roles in the pathogenesis of atherosclerosis.

$PGE_2$ modulates apoptosis predominantly through the EP2 and EP4 receptors (Chun et al., 2007; Tessner et al., 2004; Vassiliou et al., 2004), yet the role of $PGE_2$-mediated apoptosis in arterial macrophages has not been defined. Though EP2 and EP4 may have redundant functions, they employ different signaling pathways. Both EP2 and EP4 mediate signaling via cAMP-dependent protein kinase (PKA), leading to the phosphorylation of the cAMP-responsive element binding protein, which is known to regulate Bcl-2 (Regan, 2003), glycogen synthase kinase, and Bad (Fujino et al., 2003; Lizcano et al., 2000). In addition, EP4 has been reported to activate a phosphoinositide-3-kinase (PI3K) that in turn activates Akt, also known as protein kinase B (PKB). Akt inhibits cell-death pathways by directly phosphorylating and inactivating proteins involved in apoptosis, including Bad, procaspase 9, and members of the Forkhead proinflammatory family (Brunet et al., 1999; Cardone et al., 1998; Datta et al., 1997). Akt also regulates the NF-κB pathway, changing its antiapoptotic and proinflammatory functions (Madrid et al.,

2000; Ozes et al., 1999; Romashkova and Makarov, 1999). Recently, $PGE_2$ has been reported to modulate LPS-induced activation of NF-κB by a novel EP4-receptor-associated protein (Minami et al., 2008). Thus, activation of EP4 may modulate signaling pathways, regulating macrophage survival and inflammatory functions in atherosclerosis that are distinct from those of EP2.

The goal of the current study was to examine the roles of macrophage EP2 and EP4 receptors in apoptosis and atherogenesis in vivo. Given the fact that mice with targeted deletion of EP4 gene die soon after birth (Nguyen et al., 1997; Schneider et al., 2004; Segi et al., 1998), we used the approach of fetal liver cell transplantation (Babaev et al., 1999) to generate $LDLR^{-/-}$ mice chimeric for expression of EP2 and EP4 by hematopoietic cells. Our data demonstrate that the absence of EP4 in hematopoietic cells promotes macrophage apoptosis through downregulation of PI3K and NF-κB signaling pathways, and this is associated with significantly suppressed early atherosclerosis in $LDLR^{-/-}$ mice.

## RESULTS

### Generation of Mice with EP4- or EP2-Null Hematopoietic Cells

Because EP4-null mice die soon after birth (Nguyen et al., 1997; Schneider et al., 2004; Segi et al., 1998), we used fetal liver cell (FLC) transplantation to generate mice chimeric for gene deletions of EP2 or EP4 in their hematopoietic cells (Babaev et al., 1999). Eight-week-old female $LDLR^{-/-}$ mice were lethally irradiated and transplanted with female wild-type (WT; n = 12), $EP4^{-/-}$ (n = 13), or $EP2^{-/-}$ (n = 13) FLC. Twelve weeks posttransplantation, peritoneal macrophages were isolated from recipient mice, and *EP4* and *EP2* gene expression were analyzed by real-time PCR. The results indicate a complete change in the genotype to the donor types with a compensatory increase of *EP4* mRNA expression levels in $EP2^{-/-}$ cells and no changes in *EP2* gene expression in $EP4^{-/-}$ macrophages (Figures 1A and 1B). Interestingly, LPS treatment for 5 hr significantly suppressed both *EP4* and *EP2* gene-expression levels in all types of macrophages (Figures 1A and 1B).

Since *EP2* or *EP4* receptor activity may affect COX production (Fujino et al., 2003), we measured *COX-1* and *COX-2* expression in the peritoneal macrophages from $LDLR^{-/-}$ mice reconstituted with $EP2^{-/-}$, $EP4^{-/-}$, or WT macrophages. COX-1 gene and protein expression levels were similar in all three types of cells (Figures 1C and 1E). In contrast, $EP4^{-/-}$ macrophages had significantly lower levels of LPS-induced *COX-2* gene expression than WT or $EP2^{-/-}$ macrophages (Figure 3D). COX-2 protein expression was detected only in LPS-treated cells and was slightly lower (17%) in $EP4^{-/-}$ macrophages compared to WT and $EP2^{-/-}$ cells (Figure 1F).

### EP4 Deficiency in Hematopoietic Cells Suppresses Early Atherosclerosis and Increases Apoptosis in Atherosclerotic Lesions

Four weeks posttransplantation, the recipient mice were challenged with the Western diet for 8 weeks. There were no statistically significant differences in serum total cholesterol and triglyceride levels between the three groups of recipient mice reconstituted with WT, $EP4^{-/-}$, or $EP2^{-/-}$ FLC on the chow or atherogenic diets (Table S1). Analysis of the serum samples by size-exclusion chromatography revealed an accumulation of cholesterol in VLDL, LDL, and IDL fractions with no differences between mice reconstituted with WT, $EP4^{-/-}$, or $EP2^{-/-}$ FLC (Figure 2A).

To evaluate the extent of atherosclerotic lesions, we utilized two alternative methods: analysis of atherosclerotic lesions en face in whole aortas and cross sections of the aortic sinus. En face analysis demonstrated that $LDLR^{-/-}$ mice reconstituted with $EP4^{-/-}$ FLC had significantly smaller (57%) lesions compared to mice transplanted with WT or $EP2^{-/-}$ FLCs (1.5% ± 0.2% versus 2.6% ± 0.6% and 2.5% ± 0.5%, respectively; Figures 2B and 2C). Similarly, mice reconstituted with $EP4^{-/-}$ FLCs had smaller atherosclerotic lesions in the proximal aortas than mice transplanted with WT or $EP2^{-/-}$ FLC (322461 ± 34555 $\mu m^2$ versus 452708 ± 14407 $\mu m^2$ and 358179 ± 35133 $\mu m^2$, respectively; $p < 0.05$ versus WT; Figure 2D). Although there appeared to be a trend for a reduction in atherosclerosis in the $EP2^{-/-} \rightarrow LDLR^{-/-}$ mice, the difference was not statistically significant versus WT → $LDLR^{-/-}$ mice or $EP4^{-/-} \rightarrow LDLR^{-/-}$ mice.

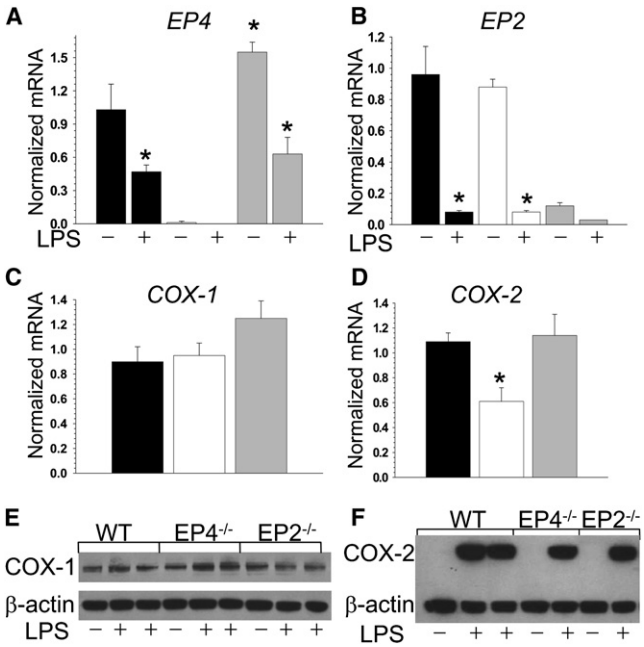


**Figure 1. *EP2* Deficiency Causes a Compensatory Increase in *EP4* Gene-Expression, and *EP4* Deficiency Suppresses *COX-2* Production in Peritoneal Macrophages**

(A–D) *EP4* (A), *EP2* (B), *COX-1* (C), and *COX-2* (D) gene-expression levels in peritoneal macrophages isolated from $LDLR^{-/-}$ mice reconstituted with WT (black), $EP4^{-/-}$ (white), or $EP2^{-/-}$ (gray) FLC and fed with the Western diet for 8 weeks. Macrophages were treated with media alone (control) or with LPS (50 ng/ml) for 5 hr. The gene-expression levels were measured by real-time PCR. Graphs represent data (mean ± SEM) with the same number (n = 3) of mice per group (*$p < 0.05$ between control and treated with LPS cells of the same group, and between WT and $EP4^{-/-}$ cells by one-way ANOVA analysis).

(E–F) *COX-1* (E) and *COX-2* (F) protein levels in peritoneal macrophages. Macrophages were treated with media alone or with LPS for 5 hr. Cell extract (20 μg/line) was resolved on 10% Bis-Tris gel and analyzed by western blot.

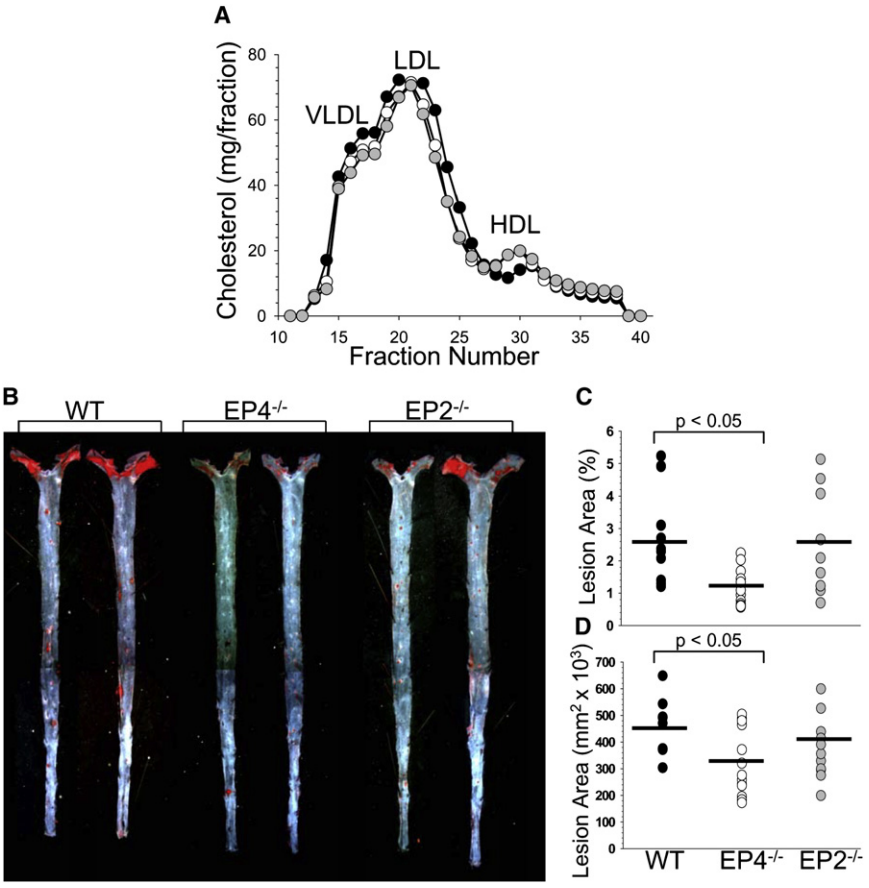


**Figure 2. EP4 Deficiency in Hematopoietic Cells Does Not Affect Plasma Lipid Levels but Significantly Suppresses Early Atherosclerosis**

(A) FPLC profiles in LDLR$^{-/-}$ mice reconstituted WT (black), EP4$^{-/-}$ (white), or EP2$^{-/-}$ (gray) FLCs. The data are represented as the average of total cholesterol of mice (n = 3 per group) reconstituted with different FLC and fed with the Western diet for 8 weeks. Fractions 14–17 contain VLDL, fractions 18–24 are IDL/LDL, and fractions 25–30 contain HDL.

(B–D) Atherosclerotic lesions in aorta en face (B) and extent of atherosclerotic lesion area in the distal (C) and proximal (D) aortas of LDLR$^{-/-}$ mice reconstituted WT (black), EP4$^{-/-}$ (white), or EP2$^{-/-}$ (gray) FLCs. Graphs represent data with different numbers (n = 12, 13, and 13, respectively) of mice of each genotype ($p < 0.05$ between control and reconstituted with EP4$^{-/-}$ FLC groups by one-way ANOVA analysis, the Tukey test).

Since $PGE_2$-dependent EP4 activation has been reported to mediate cell survival effects in dendritic cells and epithelial cells (Chun et al., 2007; Tessner et al., 2004; Vassiliou et al., 2004), we examined whether EP4 might modulate apoptosis in atherosclerotic lesions. Therefore, we stained serial sections (n = 6/aorta) from the proximal aorta with a terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL). Mice reconstituted with EP4$^{-/-}$ FLC had an increased (160%) percentage of TUNEL-positive (TUNEL+) cells in atherosclerotic lesions compared to mice transplanted with WT FLC (Figures 3A and 3B). This was accompanied by a strong trend for a decrease in the number of nuclei in the atherosclerotic lesions ($p = 0.06$) (Figure 3C) stained with macrophage-specific antibody MOMA-2 in combination with DAPI as described (Babaev et al., 2005). Mice reconstituted with EP2$^{-/-}$ FLC had a similar trend for increased numbers of TUNEL+ cells that was not statistically significant (by a one-way ANOVA). Thus, EP4 deficiency in hematopoietic cells does not affect plasma lipid levels but significantly suppresses early atherosclerosis and increases apoptosis in atherosclerotic lesions.

## Macrophage EP4 Deficiency Increases Sensitivity to Apoptotic Stimuli

Next we examined in vitro the impact of deficiency of EP2 or EP4 on macrophage sensitivity to apoptosis. To generate cells for these studies, we lethally irradiated and transplanted C57BL6 mice with WT (n = 16), EP4$^{-/-}$ (n = 14), and EP2$^{-/-}$ (n = 12) FLCs. Ten weeks posttransplantation, thioglycollate-elicited peritoneal macrophages were isolated from these mice and treated with 0.5 mM palmitic acid (PA), a stress-mediated lipotoxic factor that rapidly increases saturated lipid content in the rough endoplasmic reticulum (ER), inducing ER stress, which triggers apoptotic cell death (Borradaile et al., 2006). PA complexed with BSA treatment increased TUNEL+ cell numbers in all cell types (Figures 4B–4D) with a significantly higher percentage in EP4$^{-/-}$ macrophages than in EP2$^{-/-}$ and WT cells (Figure 4E). Remarkably, compared to WT and EP2$^{-/-}$ cells, EP4$^{-/-}$ macrophages also showed enhanced sensitivity to different stimuli, including free cholesterol loading with acetylated LDL in combination with an ACAT inhibitor (Yao and Tabas, 2001), or oxidized LDL (Figure 4F), or a specific inhibitor of IκB phosphorylation, BAY 11-7082 (20 μM), that decreases expression of NF-κB (Figure S1A). Thus, EP4 deficiency in macrophages significantly increases their sensitivity to apoptosis.

## PI3K/Akt Signaling Pathway Is Affected in EP4-Null Macrophages

To define the molecular mechanisms responsible for EP4-mediated macrophage apoptosis, we examined whether sensitivity to apoptosis in EP4$^{-/-}$ macrophages is associated with changes in signal transduction pathways. EP4$^{-/-}$, EP2$^{-/-}$, and WT peritoneal macrophages were treated with PA-BSA for 3, 6, or 18 hr, and the activity of Akt and Bad signal proteins, as indicated by phosphorylation, were analyzed by western blot. EP4$^{-/-}$ macrophages had significantly decreased levels of basal and stimulated p-Akt, whereas EP2$^{-/-}$ cells had suppression of only stimulated p-Akt (Figure 4G). In contrast, Akt levels varied insignificantly, and p-GSK3α/β and β-actin protein levels were similar in the three cell types (Figure 4G). In addition, compared to WT cells, EP4$^{-/-}$ macrophages had completely abolished phosphorylation of Bad (serine 136 and 155) after 3 hr of

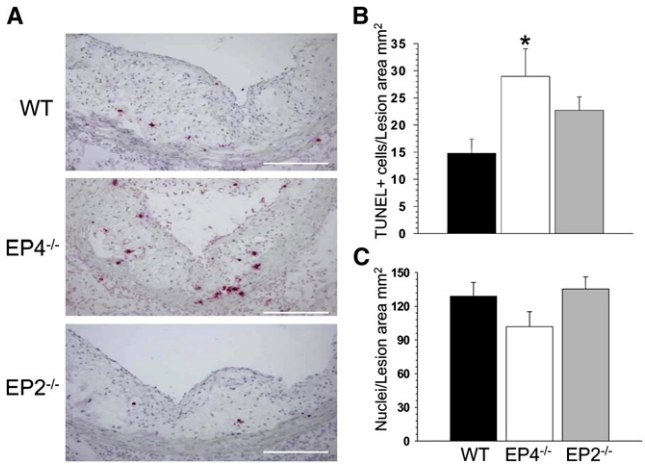


**Figure 3. EP4 Deficiency in Hematopoietic Cells Increases Apoptosis in Atherosclerotic Lesions**

(A) Distribution of TUNEL+ cells in the proximal aorta of mice reconstituted with different FLC and fed with the Western diet for 8 weeks (20x magnification). The scale bars represent 20 μm.

(B and C) Percent of TUNEL+ cells (B) and numbers of DAPI-stained nucleus cells/MOMA-2+ area (C) in atherosclerotic lesions of LDLR$^{-/-}$ mice reconstituted with WT (black), EP4$^{-/-}$ (white), or EP2$^{-/-}$ (gray) FLC and fed with the Western diet for 8 weeks. Graphs represent data (mean ± SEM) with different numbers (n = 12, 13, and 13, respectively) of mice per group (*$p < 0.05$ between mice reconstituted with WT and EP4$^{-/-}$ cells by one-way ANOVA analysis).

PA-BSA treatment (Figure 3H). Bad phosphorylation was seen after 18 hr of treatment. This suppression of p-Akt and p-Bad activity in EP4$^{-/-}$ macrophages indicates that the PI3K/Akt pathway may be responsible for the increase in apoptosis.

To test this hypothesis, we treated WT macrophages with PA-BSA alone or with different cAMP agonists, including dibutyryl cAMP or a type IV phosphodiesterase inhibitor, RO-20-1724, for 3 and 6 hr. Stimulation of the cAMP pathway with dibutyryl cAMP and, to a lesser extent, with RO-20-1724, significantly suppressed phosphorylation of Akt (Figure 5A). The dibutyryl cAMP also suppressed the phosphorylation of Bad155. Treatment with a PKA inhibitor, H89, had only minor effects on phosphorylation of Akt and Bad (Figure 5A).

In contrast, treatment of WT macrophages with a potent PI3K inhibitor, wortmannin (Wrt, 100 nM), alone or in combination with PA-BSA for 3 and 6 hr, completely abolished p-Akt activation (Figure 5B). Interestingly, more prolonged treatment with PA-BSA together with Wrt (50 nM) for 18 hr increased the number of apoptotic cells (Figure 5C) producing a dramatic cell loss (~70%), possibly due to the role of the PI3K/Akt pathway in cell attachment and migration (Shiojima and Walsh, 2002). This may explain the decrease in the percentage of TUNEL+ cells in the group treated with PA-BSA together and Wrt compared to the treatment with PA-BSA alone (Figure 4D).

Finally, to examine whether decreased Akt activation is responsible for enhanced apoptosis in macrophages, we treated WT cells with PA-BSA with or without a cell-permeable Akt inhibitor IV, which does not affect PI3K. This inhibitor acted as a proapoptotic factor and significantly (>2-fold) increased apoptotic cell numbers when combined with PA-BSA compared to PA-BSA alone (Figure 5I). The analysis of the signaling pathway demonstrated that the Akt inhibitor alone or in combination with PA-BSA completely eliminated Akt phosphorylation without affecting Akt and β-actin (Figure 5H). Together these results demonstrate that the PI3K/Akt signaling pathway is suppressed in EP4-null macrophages, leading to increased apoptosis.

## EP4 Deficiency Suppresses the NF-κB Pathway

Since Akt directly regulates the activity of the transcription factor NF-κB (Madrid et al., 2000; Ozes et al., 1999; Romashkova and Makarov, 1999), we examined whether macrophage EP4 deficiency has an impact on inflammatory and antiapoptotic genes.

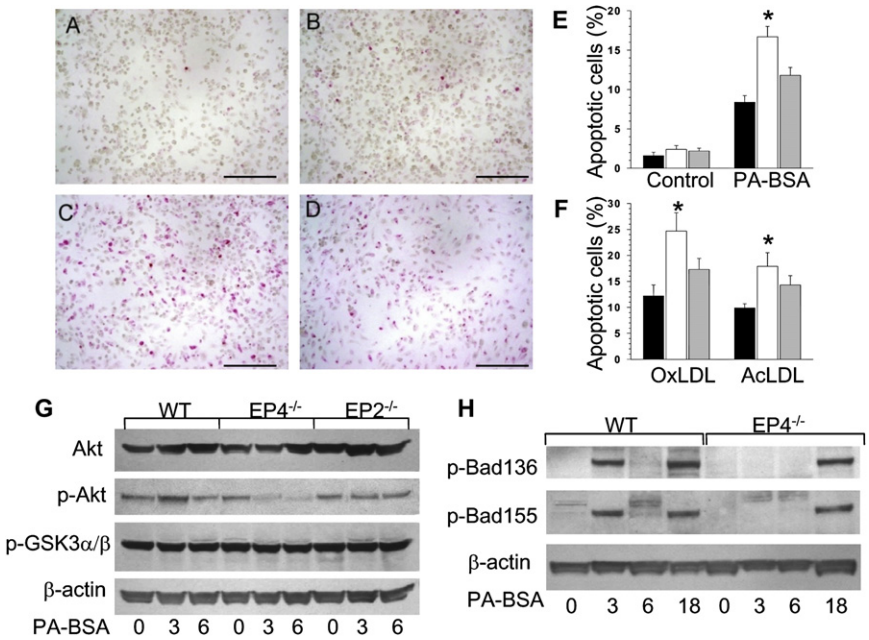


**Figure 4. EP4 Deficiency in Macrophages Increases Susceptibility to Apoptosis and Suppresses Akt and Bad Phosphorylation**

(A–D) Detection of TUNEL+ cells in untreated WT (A) and treated with PA-BSA (0.5 mM for 18 hr), WT (B), EP4$^{-/-}$ (C), or EP2$^{-/-}$ (D) macrophages (x20).

(E and F) Percent of TUNEL+ cells in WT (black), EP4$^{-/-}$ (white), or EP2$^{-/-}$ (gray) macrophages untreated or treated with PA-BSA (E), oxLDL (100 μg/ml) or AcLDL (100 μg/ml) plus an ACAT inhibitor, Sandoz 58035 (10 μg/ml) (F) for 24 hr. Graphs represent data (mean ± SEM) with the same number (n = 3) of mice per group (*$p < 0.05$ between WT and EP4$^{-/-}$ cells by one-way ANOVA analysis).

(G and H) Expression of Akt, p-Akt (serine 473), and p-GSK3α/β (H), p-Bad (serine 136 and 155), and β-actin (J) in WT, EP4$^{-/-}$ or EP2$^{-/-}$ peritoneal macrophages that were untreated or treated with PA-BSA (0.5 mM) for 3, 6, or 18 hr. Cell extract (100 μg/line) was resolved and analyzed by western blot using antibodies against the proteins as indicated.

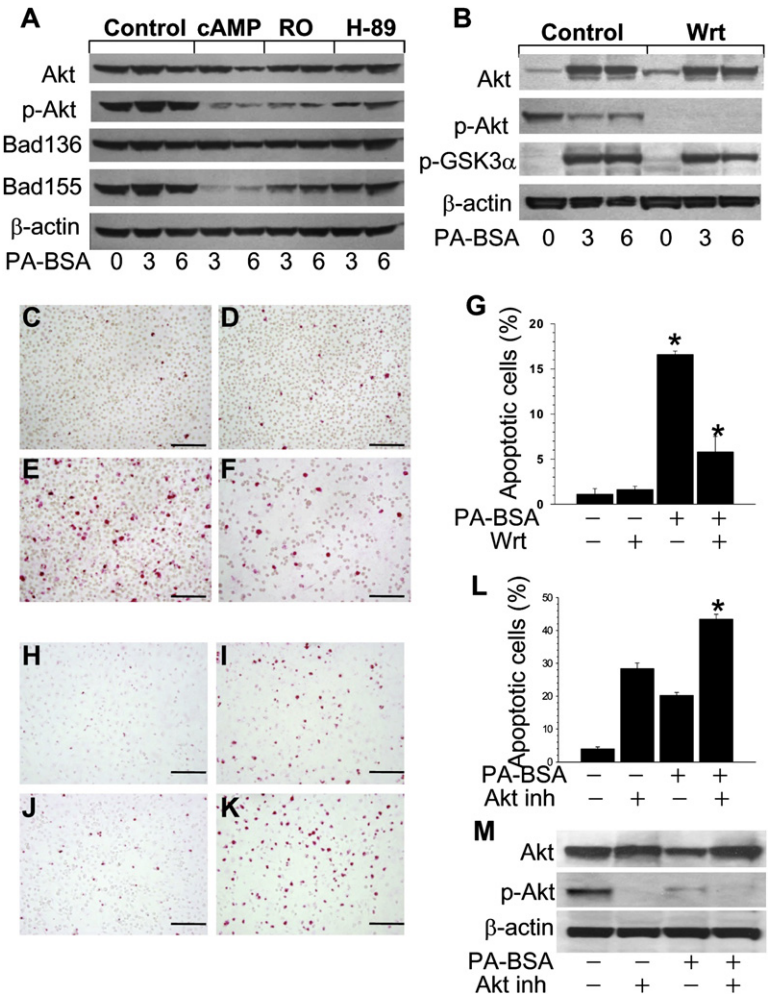


**Figure 5. Inhibition of the PI3K/Akt Signaling Pathway in WT Macrophages Accelerates Apoptosis**

(A) Expression of Akt, p-Akt (serine 473), Bad (serine 136 and 155), and β-actin proteins in WT macrophages treated with PA-BSA (0.5 mM) alone or with dibutyryl cAMP (1 mM), RO-20-1724 (100 μM), or PKA inhibitor, H89 (10 μM), for 3 and 6 hr.

(B) Expression of Akt, p-Akt, GKS3α/β and β-actin proteins in WT macrophages treated with PA-BSA alone or with wortmannin (Wrt, 100 nM) for 3 or 6 hr. Extracted proteins (100 μg/lane) were resolved and analyzed by western blot using antibodies against the proteins as indicated.

(C–F) Apoptosis in WT macrophages untreated (C) or treated with Wrt (50 nM; D) or PA-BSA (E) alone or in combination with Wrt (F) macrophages for 18 hr (x20). The scale bars represent 20 μm.

(G) Percent of apoptotic macrophages untreated and treated with Wrt (50 nM; D), PA-BSA alone or in combination with Wrt for 18 hr. Graphs represent data (mean ± SEM) with the same number (n = 3) of mice per group (*$p < 0.05$ between untreated cells and treated with PA-BSA alone and with Wrt by one-way ANOVA analysis).

(H–K) Apoptosis in WT macrophages untreated (H) or treated with the Akt inhibitor IV (10 μM; I) or PA-BSA (J) alone or in combination with the Akt inhibitor IV (K) for 24 hr (x20). The scale bars represent 20 μm.

(L) Percent of apoptotic macrophages untreated and treated with the Akt inhibitor IV (10 μM), PA-BSA alone or in combination with the Akt inhibitor IV for 24 hr (*$p < 0.05$ between cells treated with PA-BSA alone and with the Akt inhibitor by one-way ANOVA analysis, the Tukey test).

(M) Expression of Akt, p-Akt, and β-actin in WT macrophages treated with Akt inhibitor IV or PA-BSA alone or in combination for 6 hr. Extracted proteins (100 μg/lane) were resolved and analyzed by western blot using antibodies against the proteins as indicated.

Peritoneal macrophages were stimulated with LPS (50 ng/ml) for 5 hr and the expression pattern of the genes was analyzed by real-time PCR. There were no differences in the expression levels of *TNFα*, *Gro1*, and *MMP-2* genes between WT, EP4$^{-/-}$, and EP2$^{-/-}$ macrophages. However, EP4$^{-/-}$ macrophages had significantly decreased expression levels of inflammatory (*IL1β*, *IL6*, *MCP-1*, and *MMP9*), NF-κB-related (*p50*, *IKKα*, and *IKKβ*), and antiapoptotic (*Gadd45* and *Itch*) genes compared to WT and EP2$^{-/-}$ cells (Figures 6A–6I). In addition, two NF-κB-related proteins, cRel and p65, but not IκBα, were suppressed in EP4$^{-/-}$ macrophages compared to WT cells (Figure 6J). Thus, the NF-κB pathway in EP4-null macrophages is significantly suppressed.

## DISCUSSION

PGs are critical mediators of inflammation, and $PGE_2$ is a major product of activated macrophages, regulating immune responses, cytokine production, and apoptosis (Chun et al., 2007; Nataraj et al., 2001; Takayama et al., 2002; Vassiliou et al., 2004). $PGE_2$ has been implicated in the pathogenesis of atherosclerosis, and two of its four receptors, EP2 and EP4, are expressed in atherosclerotic lesions (Gómez-Hernández et al., 2006). However, the roles of macrophage EP2 and EP4 have not been previously examined in vivo. Because EP4 deficiency is lethal in mice, we used FLC transplantation to generate LDLR$^{-/-}$ mice chimeric for EP2 or EP4 gene deletions in hematopoietic cells. Here we demonstrate that LDLR$^{-/-}$ mice reconstituted with EP4$^{-/-}$ hematopoietic cells had significant decreases in atherosclerosis of the proximal and distal aortas (59% and 71%, respectively) compared to mice transplanted with WT FLC. EP2 deficiency in hematopoietic cells showed a trend for similar, but modest, effects on atherosclerosis that were not statistically significant. Since serum lipid and lipoprotein profiles did not differ between the groups, these data indicate that *EP4* deficiency in macrophages is responsible for reducing early atherosclerosis.

Although COX-2-mediated expression of $PGE_2$ has been shown to promote cell growth and suppress apoptosis in a variety of tumors (Backlund et al., 2005; George et al., 2007; Kern et al., 2006), the roles of $PGE_2$ and its receptors in modulating macrophage apoptosis in atherogenesis had not been previously examined. Recently, $PGE_2$ has been reported to mediate strong prosurvival effects via EP4 and EP2 in epithelial and dendritic cells (Tessner et al., 2004; Vassiliou et al., 2004). In our study, mice reconstituted with EP4$^{-/-}$ FLC had a striking

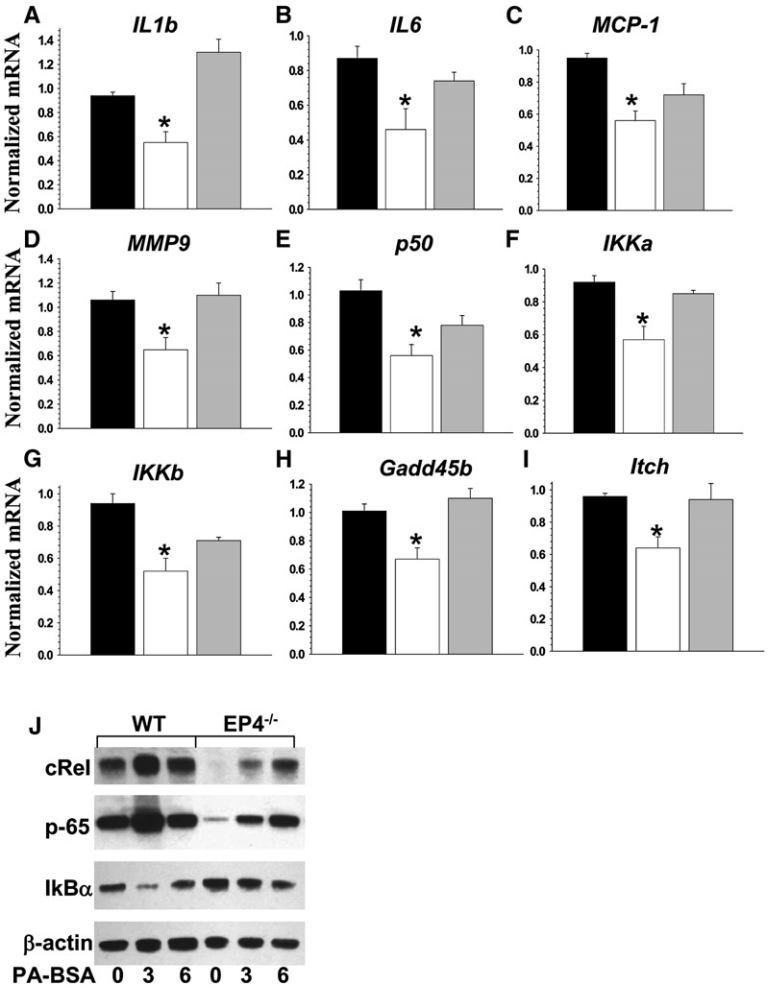


**Figure 6. *EP4* Deficiency in Macrophages Suppresses NF-κB-Related Gene and Protein Expression Levels**

(A–I) Expression levels of inflammatory (IL1β, IL6, MCP1, MMP9), NF-κB-related (p-50, IKKα, IKKβ), and antiapoptotic (Gadd45β and Itch) genes in WT (black), EP4$^{-/-}$ (white), or EP2$^{-/-}$ (gray) macrophages. The cells were treated with LPS (50 nM) for 5 hr and gene expression levels were measured by real-time PCR. Graphs represent data (mean ± SEM) of two experiments with the same number (n = 3) mice per group (*$p < 0.05$ between WT and EP4$^{-/-}$ cells by one-way ANOVA analysis).

(J) Protein expression levels in WT and EP4$^{-/-}$ macrophages treated with PA-BSA (500 μM) for 3 and 6 hr. Cell extract (100 μg/line) was resolved and analyzed by western blot. The graph represents the data of two different experiments.

increase in the number of apoptotic cells in atherosclerotic lesions compared to the recipients with WT hematopoietic cells. A similar trend for an effect of macrophage EP2 on apoptosis in atherosclerosis was observed but did not achieve statistical significance. Our results demonstrate that deficiency of EP4 promotes apoptosis in atherosclerotic lesions in vivo. Because the gene-targeted mice used in these experiments were derived from embryonic stem cells of the 129 strain and then crossed onto the C57BL/6 background, it is possible that passenger genes from the 129 strain may have contributed to the observed phenotype (Lusis et al., 2007). As pointed out by Lusis, a contribution of passenger genes is less likely when, as in our study, the mice have been backcrossed into C57BL/6 strain for ten or more generations and when the results test a prior hypothesis. Furthermore, our current data are consistent with previous studies indicating that $PGE_2$ mediates strong prosurvival effects via EP4 and EP2 in epithelial and dendritic cells (Tessner et al., 2004; Vassiliou et al., 2004) and suppressed apoptosis in a variety of tumors (Backlund et al., 2005; George et al., 2007; Kern et al., 2006).

Apoptosis plays an important role in the development of atherosclerotic lesions and plaque instability (Nhan et al., 2005; Tabas, 2005). In contrast to advanced atherosclerotic lesions characterized by an abundance of apoptotic cells due possibly to defective phagocytic clearance (Kockx, 1998; Tabas, 2005), macrophage apoptosis in the early stages of atherosclerosis can reduce plaque cellularity and volume. For example, deletion of the proapoptotic factor Bax in hematopoietic cells significantly accelerates early atherosclerosis in LDLR$^{-/-}$ mice (Liu et al., 2005). In agreement with these findings, when the apoptotic inhibitor AIM (Spα/Api6 gene) is deleted, LDLR$^{-/-}$ mice have significantly increased macrophage apoptosis and suppressed early atherosclerosis (Arai et al., 2005). In addition, mice heterozygous for macrophage colony-stimulating factor have a dramatic (<1% of controls) reduction of atherosclerosis (Rajavashisth et al., 1998). A recent report demonstrated that macrophage phospholipase Cβ3 deficiency is associated with increased apoptosis in atherosclerotic lesions and suppression of atherosclerosis (Wang et al., 2008). Our present study further extends this association between apoptosis and lesion formation by demonstrating that EP4 deficiency in hematopoietic cells significantly increases macrophage apoptosis and suppresses early atherosclerosis. Given that in advanced atherosclerotic lesions apoptosis has been implicated in promoting necrotic cell death and a greater level of plaque instability (Han et al., 2006; Thorp et al., 2008), it is reasonable to speculate that macrophage EP4 expression may modulate plaque stability in advanced lesions. Thus, EP4 and its downstream signaling pathways may provide new targets for modulating atherogenesis and plaque stability.

To examine the hypothesis that macrophage EP4 deficiency promotes apoptosis, peritoneal macrophages were challenged with PA-BSA (Borradaile et al., 2006), free cholesterol loading (Yao and Tabas, 2001), treatment with oxidized LDL, or an inhibitor of IκB phosphorylation. All these stimuli induced apoptosis at higher levels in EP4$^{-/-}$ macrophages compared to WT and EP2$^{-/-}$ macrophages. This indicates the importance of EP4 in macrophage survival signaling. Figure 7 demonstrates that both EP2 and EP4 receptors may signal through the cAMP/PKA pathway, inducing phosphorylation of a proapoptotic member of the Bcl-2 protein family, Bad at serine 155 (Regan, 2003). In addition, EP4 activates the PI3K/Akt pathway by phosphorylating Bad at two serine residues (serine 112 or 136) (Fujino

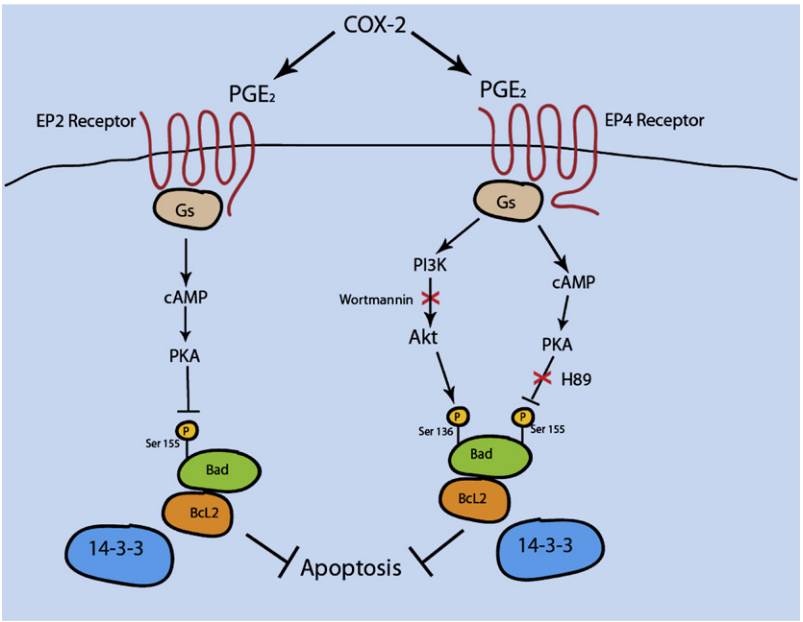


**Figure 7. A Schematic Presentation of the EP2 and EP4 Receptor-Signaling Pathways Specific for Mouse Peritoneal Macrophages Relevant to Apoptosis**

et al., 2003). In its dephosphorylated state, Bad induces apoptosis by binding antiapoptotic Bcl-2 family members, such as Bcl-xl, thereby allowing two other proapoptotic members, Bak and Bax, to form complexes, leading to the release of cytochrome *c*, caspase activation, and finally apoptosis (Wei et al., 2001). In contrast, phosphorylated Bad releases Bcl-xl and binds to the cytoplasmic anchorage protein, 14-3-3, undergoing cytoplasmic sequestration; this protects cells from apoptotic induction from different stimuli (Bergmann, 2002; Datta et al., 1999). Here we demonstrate that EP4$^{-/-}$ macrophages had significantly diminished levels of Akt phosphorylation (Figure 4H). In addition, Akt completely abolishes the activity of Bad at an early stage (3 hr) of PA-BSA treatment. Thus, genetic deficiency of EP4 in macrophages suppresses the PI3K/Akt-signaling pathway, reducing activation of Bad, which results in increased apoptosis.

Next we undertook pharmacological experiments to confirm the relevance of the PI3K/Akt-signaling pathway in mediating the impact of EP4 deficiency on macrophage apoptosis. The results strongly support the concept that EP4 deficiency suppresses macrophage survival, as treatment with cAMP agonists, but not with PKA inhibitor H89, reduces phosphorylation of Akt (Figure 4A). Similarly, only cAMP agonists suppressed phosphorylation of Bad155, which is specific for the cAMP/PKA pathway (Regan, 2003). In contrast, both the PI3K and the Akt inhibitors completely abolished p-Akt activation (Figures 5B and 5M) and dramatically increased apoptosis in macrophages (Figures 5C and 5L). Our data are supported by recent reports demonstrating that macrophages constitutively express active Akt (Liu et al., 2001) and that Akt-null macrophages are more susceptible to apoptosis (Fernández-Hernando et al., 2007). Correspondingly, the inhibition of the PI3K pathway significantly accelerates macrophage apoptosis (Liu et al., 2001), and the deletion of the PI3K p110 gamma gene significantly inhibits murine atherosclerosis (Chang et al., 2007). Together, these experiments indicate that activation of the PI3K/Akt pathway is important for macrophage survival. The suppression of this pathway in EP4-null macrophages promotes apoptosis and attenuates the development of early atherosclerosis.

In addition to an impact on apoptosis, $PGE_2$ may impact the development and fate of atherosclerotic plaques through a variety of mechanisms. For example, the PI3K/Akt pathway has been shown to be crucial in the regulation of macrophage chemotaxis and migration (Curnock et al., 2002; Hirsch et al., 2000). EP4 has been proposed to mediate $PGE_2$-induced production of MMP-9 in macrophages (Pavlovic et al., 2006), and given that apoE$^{-/-}$ mice deficient for MMP-9 gene expression have reduced atherosclerosis with decreased macrophage infiltration and collagen deposition (Luttun et al., 2004), these changes may promote plaque destabilization. Furthermore, $PGE_2$ has been proposed to impact atherosclerosis through EP4-mediated modulation of inflammation. Pretreatment of bone-marrow-derived macrophages with $PGE_2$ prior to activation with LPS results in a transitory attenuation of the early phase of production of chemokines and inflammatory cytokines (Minami et al., 2008). Interestingly, this anti-inflammatory effect is mediated by a novel EP4 receptor-associated protein that inhibits LPS-induced activation of NF-κB (Minami et al., 2008). In the absence of pretreatment with $PGE_2$, we report that activation of EP4$^{-/-}$ macrophages with LPS results in significant suppression of NF-κB-responsive genes and proteins, suggesting that macrophage EP4 may impact atherosclerosis by its effects on inflammatory cytokines such as IL-6 and MCP-1 (Figure 6). These data are consistent with evidence that activation of Akt can stimulate several signaling pathways, including NF-κB (Madrid et al., 2000; Ozes et al., 1999; Romashkova and Makarov, 1999). The NF-κB activation may induce transcription of antiapoptotic genes (including FLIP, the caspase inhibitors cIAP1/2, and Bcl-2 family members), changing the balance to prosurvival stimuli (Karin and Lin, 2002) and increasing the cells' resistance to apoptosis (Verma and Mehta, 2007). Thus, the suppression of both the PI3K/Akt and NF-κB antiapoptotic pathways may synergistically contribute to the increased apoptosis in EP4$^{-/-}$ macrophages.

Selective inhibition of COX-2 and nonselective inhibition of COX by NSAIDs have been reported to increase the risk for cardiovascular events (Bresalier et al., 2005; Grosser et al., 2006; Solomon et al., 2005). Macrophage apoptosis has been proposed to promote the formation of unstable plaques and to contribute to plaque rupture (Tabas, 2005). Given that COX-2 inhibition has been shown to promote apoptosis in a variety of tumors (Backlund et al., 2005; Kern et al., 2006), it is reasonable to speculate, based on our current findings, that COX-2 inhibition might increase apoptosis in atherosclerotic plaques by inhibiting $PGE_2$ production, and its EP4-mediated prosurvival pathways in

macrophages, providing a potential mechanism for plaque rupture and increased cardiovascular events.

In conclusion, EP4 disruption in macrophages promotes apoptosis and significantly suppresses early atherosclerosis in vivo. The genetic and pharmacological inhibition of EP4 in macrophages suppresses the PI3K/Akt-signaling pathway, reducing activation of Bad, thereby promoting apoptosis. These data indicate that macrophage EP4 expression plays a crucial role in mediating prosurvival signaling. Therefore, the $PGE_2$/EP4 pathway may provide therapeutic opportunities to modulate the development of atherosclerosis and plaque stability.

## EXPERIMENTAL PROCEDURES

### Animal Procedures

Mice heterozygous for EP4 (Schneider et al., 2004) and EP2 genes (Kennedy et al., 1999) were on the C57BL/6 background (tenth backcross). Recipient $LDLR^{-/-}$ (on C57BL/6 background) and C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained in microisolator cages on a rodent chow diet containing 4.5% fat (PMI 5010, St. Louis, MO) or a Western-type diet containing 21% milk fat and 0.15% cholesterol (Teklad; Madison, WI). Animal care and experimental procedures were performed according to the regulations of Vanderbilt University's Animal Care Committee.

### FLC Isolation, Genotyping, and Transplantation

Female $EP4^{+/-}$ (or $EP2^{+/-}$) mice were intercrossed with male $EP4^{+/-}$ (or, respectively, $EP2^{+/-}$) mice, and pregnancy was determined by the presence of a vaginal plug. On days 14—16 of gestation, embryos were isolated and their gender was determined by PCR as described (Babaev et al., 1999). To identify the genotype of $EP4^{-/-}$ FLC, a set of primers were generated (AGC GAG TCC TTA GGC TTT TAA GT, GGA GTC ACT TTC CCT TGA GAA G, and AAC GAG CCA TTT ACC ACT TGC) and used for PCR analysis (Figure S1Ba). FLCs were isolated and transplanted ($3 \times 10^6$) into lethally irradiated (9Gy) recipient $LDLR^{-/-}$ or C57BL6 mice as described (Babaev et al., 1999). FLC transplantation reconstitutes all hematopoietic cell lineages including macrophages and T and B cells (Forrester et al., 1991).

### Serum Lipids and Lipoprotein Distribution Analysis

The serum total cholesterol and triglyceride levels were determined on samples obtained from mice fasted for 4 hr as described (Fazio et al., 1997). Fast performance liquid chromatography (FPLC) was performed on an HPLC system model 600 (Waters, Milford, MA) using a Superose 6 column (Pharmacia, Piscataway, NJ).

### Analysis of Aortic Lesions

Aortas were flushed through the left ventricle, and the entire aorta was dissected for en face analysis as described (Babaev et al., 2005). Cryosections of the proximal aorta were analyzed using Imaging System KS 300 (Kontron Electronik GmbH).

### Peritoneal Macrophage Isolation and Treatment

Thioglycollate- or concanavalin-A-elicited peritoneal macrophages were isolated as described (Han et al., 2006) from mice reconstituted with wild-type, $EP4^{-/-}$, and $EP2^{-/-}$ bone marrow. Two days later, macrophages were treated with palmitic acid complexed to BSA prepared as described (Borradaile et al., 2006). Cells were treated with PA-BSA (500 μM) alone or combined with dibutyryl cAMP, RO-20-1724 (100 μM), H89 (10 mM), or wartmannin (all from Sigma), or Akt inhibitor (EMD, Calbiochem).

### Apoptosis Assessment

Serial 5-micron cryosections from the proximal aorta were fixed in 4% paraformaldehyde in PBS and treated with 3% citric acid, and apoptotic cells were detected by the TUNEL (TdT-mediated dUTP nick-end-labeling) technique using the In Situ Cell Death Detection Kit (Roche Applied Science). After treatment with Fast Red TR/Naphthol AS-NX substrate (Sigma), TUNEL-positive (TUNEL+) cells were counted in six different sections from each aorta. Peritoneal macrophages were cultured in Laboratory-Tek chamber slides (Nalge Nunc International). Cells were fixed and treated with 3% citric acid, and TUNEL+ cells were detected by the In Situ Cell Death Detection Kit.

### RNA Isolation and Real-Time PCR

Total RNA was isolated and relative quantitation of the target mRNA was performed as described (Babaev et al., 2007). The gene expression assays (Applied Biosystems, Foster City, CA) were normalized with 18S ribosomal RNA as an endogenous control.

### Western Blotting

Cells were lysed on ice with a lysis buffer (Cell Signaling Technology, Danvers, MA) containing a protease (Sigma) and phosphatase (Pierce) inhibitor cocktails. Protein concentrations were determined with the DC Protein Assay Kit (Bio-Rad Laboratories). Lysates (20 or 100 μg/*lane*) were resolved by NuPAGE Bis-Tris elecrophoresis (Invitrogen) and transferred onto polyvinylidene difluoride nitrocellulose membranes (Amersham Bioscience). Blots were probed with rabbit antibodies to Akt, p-Akt, pGSK3α/β, p-Bad136, p-Bad155 (all from Cell Signaling), c-Rel, NF-κB p65 and IκBα (all from Santa Cruz Biotechnology), β-actin antibody (Abcam, Inc. Cambridge, MA), and goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Upstate Cell Signaling, Lake Placid, NY). Proteins were visualized with ECL western blotting detection reagents (GE Healthcare) on X-ray films. Protein levels were quantified by densitometry normalizing to β-actin.

### Statistical Analysis

The statistical differences in mean serum lipids and aortic lesion areas between the groups were determined by a one-way ANOVA test using SigmaStat V.2 (SPSS Inc., Chicago, IL).

## SUPPLEMENTAL DATA

Supplemental Data include one table and two figures and can be found online at http://www.cell.com/cellmetabolism/supplemental/S1550-4131(08)00289-1.

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